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Genetic Anomalies in Mammalian Germ Cells and Their Significance for Human Reproductive and Developmental Risk

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The induction of heritable mutations in germ cells represents a potential health concern. This paper highlights data from mouse germ-cell mutagenesis studies that have implications in the assessment of reproductive and developmental risks. The paper discusses the developmental and reproductive consequences of induced chromosomal damage (structural rearrangements and numerical anomalies) and describes environmental agents that have been shown to produce such anomalies. Additionally, factors that influence the yield of genetic damage are addressed. Studies showing that the various germ-cell stages vary in their susceptibility to the induction of genetic damage are summarized. Of the chemicals evaluated in the male mouse, most appear to have their predominant or strongest effect on post-stem-cell stages. The differences between males and females in the susceptibility to mutagens is examined. Recent studies have shown that the female may be uniquely sensitive to certain mutagens. Finally, an important aspect of mutagenic risk is not only effects induced in developing germ cells but also the effects of environmental agents during the period from fertilization through the zygote and the two-cell embryo. Recent work in the mouse has demonstrated that exposure during these early developmental stages leads to high frequencies of external and visceral fetal malformations, as well as mid-to-late gestational death.

Introduction

Environmental exposure of the human population to DNA- and chromosome-damaging agents is an important toxicological problem. Mutagens may contribute to somatic-cell diseases, such as cancer, or may cause an increased incidence of genetic diseases in future generations. Because genetic damage in germ cells can lead to reproductive impairment and developmental anomalies, knowledge of induced germ-cell mutagenesis is relevant to the assessment of reproductive and developmental hazards in humans from exposure to environmental contaminants.

No environmental agent, including radiation, has been shown to produce heritable germ-line mutations in humans. The lack of human evidence relates to the rarity of gene mutations, the small fraction of human genes that are currently useful as markers in estimating germ-cell mutation, human genetic variability, small numbers of offspring, and long generation times. Further, only disorders caused by dominant mutations, some sex-linked recessive disorders, and chromosome-based disorders can be detected in the first generation after their occurrence. Many human diseases are inherited (*1*), and it is generally recognized that most newly appearing mutations that are phenotypically expressed are in some ways deleterious (*2*). A large number of synthetic chemicals have been found to induce genetic damage that is transmitted to the offspring of laboratory animals, primarily the mouse (*3*). This is the most important justification for the concern that chemical mutagens may contribute to the genetic disease burden in humans.

Assessment of genetic risk and the possible reproductive and developmental consequences to humans from exposures to environmental mutagens requires accurate identification and quantification in experimental systems of the types of genetic damage that can be produced in the germ line and transmitted to the conceptus. The types of damage considered in genetic risk assessment are gene mutations (dominant, semidominant, and recessive), small deletions, structural chromosomal anomalies (reciprocal translocations, inversions, duplications, etc.), and numerical chromosomal anomalies (whole chromosomes missing or in excess).

This paper is not intended to be a complete review but instead considers certain aspects of germ-cell mutagenesis that have implications in the assessment of reproductive and developmental hazards associated with exposure to environmental agents. This paper focuses on several

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topics: *a*) the impact of induced chromosomal damage (structural rearrangements and numerical anomalies), *b*) the relative susceptibility of the various germ-cell stages to the induction of heritable or transmissible genetic damage, *c*) gender differences in the susceptibility to mutagens, and *d*) developmental responses of zygotes and early cleavage embryos to mutagenic exposures.

Chromosomal Anomalies Are Associated with Reproductive and Developmental Defects

Chromosomal anomalies constitute a significant medical problem, and thus the chromosomal constitution of human germ cells is of intense interest in such fields as genetics and mutagenesis, epidemiology, human reproduction and development, and health risk assessment. Approximately 1 in 200 live births has a detectable major structural or numerical chromosomal disorder (*4*). Down's syndrome (trisomy 21), for example, is the most common chromosome anomaly among live births: it is at least twice as common as the single-gene trait cystic fibrosis, and 20 times more common than the single gene traits muscular dystrophy or phenylketonuria. Chromosomal anomalies are, in fact, more common in human beings than in any other species (*5*). They form the largest known cause of first trimester loss. The basis for this high frequency is unclear.

Numerical Chromosome Anomalies

Abnormal chromosome numbers (heteroploidy) is an important genetic hazard to humans (*6–8*). More than 70% of the chromosomal errors associated with early spontaneous abortion and nearly half of all those detected among newborns are aneuploids [i.e., chromosome number is not an exact multiple of the basic number in the genome (*4,7*)]. Additionally, polyploidy (chromosome state in which all chromosomes are represented more than twice), particularly triploidy, is also common in humans, with an estimated frequency of 26% in aborted fetuses (*7*). Given the social and financial burdens posed by the consequences of numerical chromosome errors in human beings, it is unfortunate that the reasons for the human incidence of heteroploidy and the mechanisms by which it arises remain mostly unknown. It is also unfortunate that relatively little testing has been accomplished to detect the ability of environmental agents to induce heteroploidy (*9*) compared to other genotoxic end points (e.g., gene mutation and chromosomal breakage).

The absence of sufficient data on chemically induced heteroploidy in mammalian germ cells is due to the lack of dependable and reliable test approaches. In rodents, investigators have relied mostly on cytogenetic analysis of germ cells to determine aneuploidy at the gametic level. The utility of cytogenetic analysis may be increased by several molecular cytogenetic approaches under development that may allow for a more reliable and rapid method of detecting germ-line aneuploidy than is now possible through conventional cytogenetic analysis. For example, the localization of kinetochores in micronucleated cells using immunofluorescent-kinetochore staining has been developed for identifying aneuploidy-inducing agents in cultured somatic cells (*10*). This approach is being pursued in mouse spermatids (*11*). Additionally, the technique of fluorescent *in situ* hybridization using DNA probes is a promising approach that may have application for detecting aneuploidy in both human and rodent germ cells (*12,13*).

Both ionizing radiation and chemicals have been shown to increase aneuploidy in mammals (*6*). Radiation probably causes aneuploidy by breaking chromosomes. Chemicals also may act in this manner. Alternatively, chemicals may also act on the spindle or the segregation process. It is widely held that chromosome pairing, synapsis, and crossing over are important events for normal disjunction and segregation and that aberrant synapsis/recombination could lead to aneuploidy (*14–16*). For example, studies in Drosophila (*17*), mice (*18*), and humans with trisomy 21 (*19*) imply that a low level of recombination results in chromosomal nondisjunction.

Because of the substantial contribution of aneuploidy to human infertility, reproductive failure, and congenital malformation, there is a clear need to study the mechanisms of aneuploidy production and transmission in experimental mammals and to understand how and to what extent aneugenic agents exert their effect on mammalian species including humans. An understanding of the basic mechanisms and components involved in chromosomal segregation is essential for understanding how chemicals might interfere with this process.

Chromosomal Rearrangements

Physical rearrangements of the genetic material can lead to profound biological consequences. Rearranged chromosomes can give rise to abnormal meiotic chromosome pairing, which in turn can lead to infertility or low fertility. Structural rearrangements, particularly reciprocal translocations, have been associated with infertility, low fertility, or spontaneous abortion (*20,21*). In both humans and mice, carriers of translocations have been associated with developmental effects such as neurological and skeletal disorders (*22–24*). For example, a male translocation carrier that showed distinctive neurological symptoms was found among the offspring of male mice treated with the mutagen triethylenemelamine (*22*). Although carriers of balanced translocations (i.e., no genetic material has been lost or added through the rearrangement) may appear phenotypically normal with the exception of some effect on fertility, they may produce offspring with developmental abnormalities. The genetic burden and medical consequences of this aneuploidy is increased due to the transmissibility of the aneuploidy to subsequent generations.

The developmental consequences of chromosome imbalance produced by translocations in the mouse have been recognized for over 50 years (*25*). These imbalances are the result of translocated chromosomes going through meiotic segregation and producing gametes with unbalanced chromosomal constitutions. Several translocation mouse stocks have been studied, and a number of specific malformations have been identified (*26*). In a recent study by Rutledge et al. (*27*), for example, a high incidence of developmental anomalies, such as exencephaly and cleft palate, was found among fetuses sired by carriers of translocations who appeared phenotypically normal except for semisterility. These male translocation carriers were derived from male mice treated with the mutagen methylenebisacrylamide. In the offspring of these carriers, some of the malformations observed in the conceptuses were associated with specific duplication deficiencies caused by the segregation of the translocated chromosomes. Study of the reproductive consequences of multiple stocks with induced balanced translocations revealed that 17% of stocks had excess phenotypic anomalies analogous to liveborn humans with birth defects and 42% had excess late gestational deaths (which would equate in humans to excess mid-pregnancy and third trimester miscarriages and their attendant morbidity). In humans, it has been observed that carriers of balanced translocations may be at risk of having chromosomally unbalanced children that are congenitally malformed and mentally retarded (*28,29*).

More than a dozen chemicals have been shown to induce transmissible translocations in the mouse (*3,30*). Most of these studies have been conducted in the male. Several of the chemicals shown to cause heritable translocations are of environmental interest. For example, mouse heritable translocation data on ethylene oxide, which is used as a sterilant, fumigant, and as an intermediate in the production of other chemicals, has been used as a model for quantifying risks to the human germ line (*31*). Inhalation exposure to ethylene oxide produced high frequencies of heritable translocations in male mice with a steep nonlinear dose–response curve (*32*). Ethylene oxide also induces gene mutations (i.e., dominant visible and electrophoretic) in germ cells (*33*). Acrylamide, methylenebisacrylamide, ethylenebisacrylamide, and other industrial chemicals, have been shown to induce heritable translocations (*3,27,34*). Additionally, several chemotherapeutic agents, such as cyclophosphamide, chlorambucil, mitomycin C, procarbazine, and triethylenemelamine, have been shown to induce translocations and specific locus mutations (*3*).

Germ-Cell Stages Vary in Their Susceptibility to the Induction of Transmissible Genetic Damage

Chemicals show differences in susceptibility of various spermatogenic stages to the induction of heritable damage (*35,36*). Of the chemicals evaluated to date, all appear to have their predominant or strongest effect on post-stem-cell stages (i.e., spermatocytes, spermatids, spermatozoa) in producing transmitted germ-line translocations (*30,32,34*). Unlike radiation, no stem-cell induced effects have been identified in the mouse dominant lethal or heritable translocation test [(*38*); W. M. Generoso, personal communication]. For induced specific-locus mutations, chemicals have been found to affect both stem cells and post-stem-cell stages (*36*).

Although risk to agents inducing post-stem-cell damage would be a function only of recent exposure (because of their relatively short life span) and should recede upon termination of exposure, many human exposures are repeated or continuous in both industry and the environment. Thus, heritable damage induced in post-stem cells is important for the estimation of genetic hazards. In instances, however, in which exposure is not continuous or repeated, it may be possible to greatly reduce the genetic hazard by avoiding conception for a designated time after exposure. Unlike post-stem cells, damage induced in stem cells would become permanent sources of mutation- or translocation-bearing sperm, and such damage would accumulate throughout the exposure history of the individual. The affected individual would be at some level of increased risk for his entire reproductive life even after exposure had ceased. The sensitivity of male germ-cell stages to environmental mutagens and the application of this information has been illustrated recently in a quantitative estimation of genetic risk associated with the induction of heritable translocations by ethylene oxide (*31*).

Unlike the male, little is known about the sensitivity of female germ-cell stages to environmental mutagens. Radiation studies indicate differential susceptibility of the newborn mouse ovary to cell killing and mutation induction, which is in various stages of prophase of meiosis, to that of the adult, which contains oocytes in follicles of varying degrees of maturity (*37*). For both radiation and chemicals, it appears that there is differential susceptibility of ovarian follicles at various stages (*39,40*). Further, it is clear from what information exists on mutation induction that there are differences in the sensitivity between female and male germ cells. The importance of female germ-cell mutagenesis studies will be described in the next section.

Female and Male Mice Respond Differently to Mutagenic Agents

Because of the difficulties encountered in studying effects in mutagen-treated female mice (e.g., toxic effects on pregnancy, breeding problems), little information is available on the induction of genetic damage. Because of the differences in male and female gametogenesis (e.g., cellular morphology, cellular kinetics, biochemistry, chromatin structure), the male is unlikely to be a reliable surrogate for predicting female genetic risk to a particular agent. Recent dominant lethal studies indicate that certain chemicals can only be detected as mutagenic in the female but not in the male (*40–42*).

The mutagens platinol, adriamycin, hycanthone, and bleomycin appear to induce dominant lethal mutations only in female mice (*40–42*). Bleomycin caused the strongest response, producing up to 90% dominant lethals at about one-fifth of the maximum-tolerated dose (10 mg/kg). The magnitude of response observed with bleomycin is greater than that observed with other agents, including ionizing radiation.

Interpretation of results from genotoxicity testing in the female can be complicated by the need to distinguish between embryonic death caused directly by genetic effects of the chemical on the oocytes and embryonic loss resulting indirectly from maternal toxicity. It is thought that these dominant lethal effects are the result of genetic damage and not of maternal toxicity because reciprocal zygote transfer experiments with adriamycin and platinol were indicative of genetic damage to the oocytes (*41*). Furthermore, chromosomal aberrations were observed at the first cleavage of zygotes obtained from females treated with adriamycin, platinol, bleomycin, and hycanthone (*40–42*).

Although these agents have no detectable dominant lethal effects in males, it should not be assumed that these agents are inactive in causing genetic damage in the male. Bleomycin reportedly induces low levels of chromosomal damage in spermatocytes (*43*) and in spermatogonia in male mice (*44–46*). So although no dominant lethal mutations are detected in the male, there still may be a low level of damage being induced. Nevertheless, these data still indicate that the female is at a much higher level of risk than the male.

Sudman et al. (*40*) suggested that the state of chromatin condensation in the germ cells of female mice may be involved in the increased vulnerability to these mutagens. The majority of germ cells in adult female mice are arrested in the dictyate (diffuse diplotene) stage of meiosis in which the chromatin is in a diffuse state, while in adult males all stages of spermatogenesis are present simultaneously, and the chromatin in most germ-cell stages is generally more condensed (*47*). The diffuse state of chromosomes in the resting dictyate oocyte stage would allow intercalation between base pairs and alkylation of bases to occur, whereas the more condensed state of chromatin in male germ cells may impede the interaction of these agents with DNA. It is generally agreed that adriamycin and hycanthone are intercalating agents (*48*). It is postulated that bleomycin acts as an intercalator (*49,50*). On the other hand, in the case of platinol (a bifunctional alkylating agent), it was postulated that specific base sequences, which are preferential targets, are more accessible for DNA biding in the female chromatin than in the male chromatin (*41*). There are other possibilities that may contribute to the increased susceptibility of the female compared to the male such as differences in the transport and metabolism of the agent in male as opposed to female gonads.

In summary, results of studies that demonstrate the existence of agents that pose a greater genetic hazard to female mice than to male mice emphasize the importance of developing female models to evaluate genetic/reproductive/developmental risk from exposure to environmental agents. In developing these models, several questions need to be addressed. What classes of chemicals produce female-specific responses? What is the underlying basis for the increased vulnerability of the oocyte? The mechanism involved must be a result of properties unique to female gametogenesis. The relationship of chromatin condensation and DNA binding in male and female mouse germ cells would provide some understanding regarding the plausibility of this hypothesis concerning chromatin condensation and susceptibility to DNA damage. Studies addressing the etiology and mechanisms involved in these effects will contribute to the development of approaches for characterizing female gametic risk associated with exposure to mutagens.

Developmental Anomalies Arise from Mutagen Treatment of Zygotes and Early Cleavage Embryos in the Mouse

In addition to evaluating risk associated with exposure to developing germ cells, an important aspect of mutagenic risk is the effects of environmental mutagens during the period from fertilization through early zygotic and cleavage stages. Although it is generally believed that exposure of the one-cell zygote and early cleavage stages to a mutagen can result in death of the zygote or no response, recent studies have clearly demonstrated that mutagen exposure during early embryogenesis can result in other manifestations of developmental toxicity as well as malformations.

Mouse studies conducted at the Oak Ridge National Laboratory (Oak Ridge, Tennessee) have shown that exposure of zygotes to certain chemical mutagens leads to high frequencies of external and visceral fetal malformations, as well as late-gestational death (*51–55*; Kimmel et al., in preparation). The fetal malformations induced generally bear close resemblance to common sporadic human birth defects that are of unknown etiology. Most of the chemicals evaluated to date have been alkylating mutagens. Some of these agents (e.g., ethylene oxide, ethyl methanesulfonate, diethylsulfate, dimethylsulfate, and mitomycin C) were found to produce both fetal malformation and mid- to late-gestational death, while others (e.g., chlorambucil, acrylamide, methylmethane sulfonate, ethylnitrosourea, triethylenemelamine) only elicited an increase in malformations (*51,52,55*). This suggests that the type of mechanisms that lead to various types of fetal anomalies may differ from one mutagen to another. In general, the period of vulnerability to these short-lived mutagens appears to begin approximately around the time of sperm penetration, with the most pronounced effect occurring near the early pronuclear stage and disappearing by the first cleavage stage. The temporal effects of these mutagens suggest different mechanisms for the induced fetal death and malformation but do not necessarily imply a conventional mutational mechanism (discussed later). Transplantation experiments involving reciprocal transfer of pronuclear zygotes within hours after treatment with ethylmethane sulfonate suggest that maternal toxicity is not a factor in producing dysmorphic fetuses (*41*).

Among the alkylating agents evaluated, ethylene oxide (EtO) has been the most studied. Recently, the dose–response relationship between early zygotic exposure to EtO and prenatal development was evaluated (Kimmel et al., in preparation). Six hours after mating, female (C3H ×

C57BL)F_1 mice were exposed to several doses of inhaled EtO (1200, 900, or 600 ppm) for a duration of 1.5 hr. A high incidence of mid- and late-gestational deaths and malformations were induced at the two highest concentrations of EtO. The malformations observed included limb defects, cleft palate, abdominal wall defects, hydrops, exencephaly, eye defects, and growth retardation. The effect of EtO on skeletal development was evaluated at the 1200, 900, and 600 ppm doses. EtO was found to significantly reduce the degree of ossification in treated offspring in a dose-related manner and increase the incidence of skeletal variants, particularly in the sternal and cervical regions, a spectrum of skeletal anomalies differing from treatment during organogenesis (*54*). The lowest exposure of EtO, 600 ppm, did not elicit an apparent difference from the control in the incidence of death and malformation (external and visceral effects). The slope of the dose–response curve for EtO under the above exposure scenario appears steep and implies an apparent threshold of response.

Although the basis for mutagen-induced zygote responses is not known, current data suggest multiple mechanisms. In the case of ethylnitrosourea (ENU), there is evidence that gene mutations might be a factor for the observed induced fetal anomalies. Russell et al. (*56*) found that early zygote stages in the mouse were sensitive to the ENU induction of specific locus mutations. The frequency of specific-locus mutants resulting from exposure of early zygote stages was about 7-fold higher than the mutant frequency that occurred as a result of spermatogonial stem-cell exposure to ENU. ENU exposure of early mouse zygotes also induces a high frequency of transmitted skeletal anomalies (*57*). These results raise the concern that early-stage zygotes may be at higher risk to certain mutagens than developing germ cells. Interestingly, Russell and Bangham (*58*) found that the maternal genome in the mouse zygote (near sperm entry and early pronuclear stages) is more sensitive to ENU mutagenesis than the paternal genome. The basis for this difference is unknown. For EtO and ethyl methanesulfonate (EMS), the induced malformations do not appear to be attributable to chromosomal rearrangements, small deletions, or gene mutations (*59*). Conventional mutagenic mechanisms, therefore, are not sufficient in explaining the EtO- and EMS-induced effects.

Recent studies conducted by Generoso (*60*) on the chemotherapeutic agent 5-azacytidine suggest a different mode of action postulated for the alkylating agents. Unlike the alkylating mutagens, such as EMS and EtO, the response for malformation significantly increased when exposure was 25 hr after mating. In the mouse this would correspond to approximately the time when embryonic genes are beginning to be switched on (*61*). From these data, it can be speculated that induced fetal maldevelopment may also occur as the result of induced epigenetic changes in early zygote stages (e.g., disruptions in the finely tuned program of gene expression that influences early embryonic development). Azacytidine is a nucleoside analog that can selectively activate eukaryotic gene expression. It is thought to act by inhibiting enzymes that methylate cytosine residues in eukaryotic DNA (*62*).

In summary, an important aspect of germ-cell mutagenesis in producing adverse reproductive and developmental effects includes exposures not only during critical periods of gametogenesis but also near fertilization and during early developmental stages, thus extending the temporal period of susceptibility and raising the important possibility of new mechanisms by which mutagenic agents interfere with development. The similarity of the phenotypic consequences further suggests that some of the mechanisms may be associated with the largest etiologic group of human developmental anomalies.

Concluding Remarks

This paper has attempted to point out several themes in the area of germ-cell mutagenesis that have important implications for reproductive and developmental toxicology. Because of the impact of genetic damage on normal reproduction and development, testing should be pursued to identify germ-cell mutagens, clastogens, and aneugens. Although chromosomal anomalies play an important role in producing developmental defects, the mechanistic basis for the vast majority of human developmental defects remains unknown. The observations of zygote-derived developmental anomalies in mutagen-exposed pregnant mice is of major interest in chemical safety evaluation and in studying the etiology of congenital abnormalities. Not only do these observations raise concerns regarding the vulnerability of human zygotes and early-cleavage embryos of mothers exposed to environmental chemicals, but research defining the biological factors that affect the induction of zygote-derived developmental anomalies may prove helpful in understanding the large group of fetal malformations in humans for which the etiology is still unknown. Genetic hazards associated with mutagen exposure to females cannot be neglected in light of recent findings of female-specific responses in the dominant lethal test. Finally, although defined windows of susceptibility to induced genetic damage have been observed in experimental studies, if human exposures are continuous or repeated, it is likely that there will be exposure during the window of sensitivity. Thus, postspermatogonial stem cell induced mutations are of significance in assessing genetic/reproductive/developmental risk.

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